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Experimental Model for Cartilage Tissue Engineering to Regenerate the Zonal Organization of Articular Cartilage

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Summary

Objective: Regeneration of the zonal organization of articular cartilage may be an important advancement for cartilage tissue engineering. The first goal of this study was to validate our surgical technique as a method to selectively isolate chondrocytes from different zones of bovine articular cartilage. The second goal was to confirm that chondrocytes from different zones would have different proliferative and metabolic activities in two-dimensional (2-D) and 3-D cultures. Finally, to regenerate the zonal organization, we sought to make multi-layered constructs by encapsulating chondrocytes from different zones of articular cartilage.

Design: Cartilage slices were removed from three (upper, middle, and lower) zones of articular cartilage of young bovine legs. Histology and biochemical composition of the cartilage slices were analyzed to confirm that they had been obtained from the proper zone. Growth kinetics and gene expression in monolayer culture and matrix formation in photopolymerizing hydrogels were evaluated. Multi-layered photopolymerizing hydrogels were constructed with chondrocytes from each zone of native cartilage encapsulated. Cell viability and maintenance of the cells in the respective layer were evaluated using the Live/Dead Viability kit and cell tracking protocols, respectively. After 3 weeks, the multi-layered constructs were harvested for histologic examination including immunohistochemistry for type II collagen.

Results: Analysis of histology and biochemical composition confirmed that the cartilage slices had been obtained from the specific zone. Chondrocytes from different zones differed in growth kinetics and gene expression in monolayer and in matrix synthesis in 3-D culture. Cells encapsulated in each of the three layers of the hydrogel remained viable and remained in the respective layer in which they were encapsulated. After 3-week culture, each zone of multi-layered constructs had similar histologic findings to that of native articular cartilage.

Conclusion: We present this as an experimental model to regenerate zonal organization of articular cartilage by encapsulating chondrocytes from different layers in multi-layered photopolymerizing gels.

Key words: Articular cartilage, Zonal organization, Chondrocyte, Photopolymerizing hydrogel.

Introduction

Articular cartilage has a limited capacity to repair itself[1–3]. No current treatment modalities restore injured cartilage to the original tissue with normal functional and structural properties[4,5]. As a multidisciplinary effort, cartilage tissue engineering has been explored to develop better treatment options for this difficult condition[1,2,6–9]. A great deal of effort has been focused on introducing cells, with or without a scaffold, into the injured or diseased site to replace the damaged cells and augment the production and remodeling of extracellular matrix production[1,7–13]. Of the numerous efforts involving cell-based therapies, autologous chondrocyte transplantation has been reported with promising clinical and experimental results[5,7,9]. However, there have been other experimental studies questioning the benefit of autologous chondrocyte transplantation for the regeneration of injured cartilage[1,6,10].

The current general paradigm of tissue engineering strategies is to mimic as closely as possible the native environment and structure of a tissue in order to encourage the restoration of its structure and function[3,11–13]. Native articular cartilage has a zonal organization as evidenced by different cell morphologies, cell arrangements, biochemical compositions, and mechanical properties[14–16]. This zonal organization is important for the normal physiologic role of articular cartilage[14,15,17–23]. Chondrocytes are responsible for the development and maintenance of the zonal architecture of articular cartilage[17–20,22]. Chondrocytes from different zones have different metabolic activities even after being released from cartilage and cultured in three-dimensional (3-D) system[18,19]. In rebuilding the zonal organization of cartilage, one would ideally want to use the building blocks that make up the original structure. It is conceivable that chondrocytes from each zone would be the optimal cells to rebuild that specific zone.

A variety of synthetic polymers and natural substrates have been used as scaffolds in tissue engineering[13,24,25].

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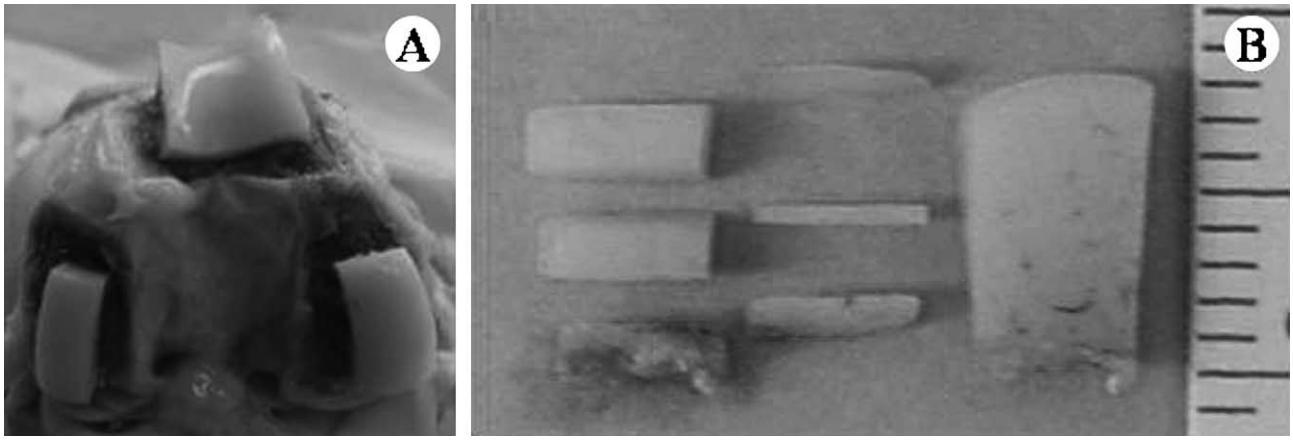

Fig. 1. Photographs demonstrating the surgical technique to harvest cartilage slices from the different zones. Cartilage was taken from the patellofemoral groove and distal femoral condyles (A). The top 10%, central 10%, and bottom 10% of the excised tissue were harvested to isolate the upper, middle, and lower cartilage (B). Abbreviations: U—upper; M—middle; and L—lower.

Hydrogels provide numerous advantages including high, tissue-like water content, efficient transport of nutrients and waste, and the powerful ability to effectively and uniformly encapsulate cells[24,25]. Photopolymerization is one method to form a hydrogel through reactions in which polymer solutions with appropriate reactive groups cross-link to form a gel in the presence of a photoinitiator and light[24,26,27]. Photopolymerization provides effective spatial and temporal control over hydrogel formation[26,27]. Photopolymerizing hydrogels are used in numerous biomedical applications[24]. Previous studies using this photopolymerizing poly(ethylene glycol)-based polymer system have demonstrated the ability to encapsulate chondrocytes in hydrogels to engineer cartilage-like tissue *in vitro* and *in vivo*[26–28].

Despite the importance of zonal organization for the normal function of articular cartilage, most previous studies purported to regenerate cartilage with a homogenous structure without zonal variation[7,9,29]. Few previous studies attempted to use chondrocytes from different zones to regenerate the zonal architecture[30,31]. In an effort to create zonal organization in engineered cartilage, we have sought to take advantage of the ability to temporally and spatially control the photopolymerization reaction[26,27,32]. Towards this end, we have established a method in our laboratory to isolate chondrocytes from the different zones (upper, middle, and lower) of young bovine articular cartilage while minimizing contamination by cells from adjacent layers. In this paper, we present our method of isolating chondrocytes from different zones and verify that these cells from different layers have different behaviors in 2-D and 3-D cultures. Finally, we demonstrate an experimental model to regenerate the zonal organization of articular cartilage by encapsulating chondrocytes from different zones into multi-layered photopolymerizing gels.

Methods

SURGICAL DISSECTION TO ISOLATE CARTILAGE SLICES FROM DIFFERENT ZONES

Cartilage slices were taken from the patellofemoral groove and femoral condyles of six legs from three 5- to 8-week-old calves. To obtain cartilage blocks with similar shape, only central areas were removed from the patellofemoral groove, medial femoral condyle, and lateral femoral condyle [Fig. 1(A)]. In order to facilitate defining the three zones, cartilage was taken *en bloc* from the subchondral bone. The thickness of the cartilage block ranged from 2 to 6 mm depending on the joint area. To minimize the contamination by cells from adjacent zones, only the top 10%, central 10%, and bottom 10% were taken from the upper, middle, and lower zones, respectively. Briefly, the top 10% (200–600 μm) was first taken from the cartilage block using a surgical blade. After the following 30% was discarded, the next 10% (200–600 μm) was taken from the middle zone. After the following 30% and the most bottom 10% including remaining subchondral bone were discarded, the bottom 10% (200–600 μm) was harvested for the lower zone.

HISTOLOGIC EVALUATION OF CARTILAGE BLOCK AND CARTILAGE SLICES FROM DIFFERENT ZONES

To confirm that cartilage slices were obtained from the specific zone, histologic evaluation of the cartilage taken *en bloc* and cartilage slices from three layers was performed. Formalin fixed, paraffin embedded specimens were sectioned and stained with safranin-O/fast green and Masson's trichrome using standard histological procedure.

BIOCHEMICAL ASSAY OF CARTILAGE SLICES FROM DIFFERENT ZONES

Biochemical compositions of the excised cartilage slices were determined by DNA assay, glycosaminoglycan (GAG) assay, and collagen assay. Wet weights (ww) and dry weights (dw) were obtained from the cartilage slices (N=9, from three different animals) before and after 48 h of lyophilization. The dried specimens were digested in 1 ml of papain solution (125 μg/ml papain; Worthington Biomedical Corporation, Lakewood, NJ, USA), 100 mM phosphate buffer, 10 mM cysteine, 10 mM EDTA, pH 6.3, for 18 h at 60°C. The DNA content (ng of DNA/mg dw of the cartilage slice) was determined using Hoechst 33258[33]. GAG content was estimated by chondroitin sulfate using dimethylmethylene blue dye[34]. Total collagen content was determined by measuring the hydroxyproline content of the specimens after acid hydrolysis and reaction with

p-dimethylaminobenzaldehyde and chloramine-T using 0.1 as the ratio of hydroxyproline to collagen[35]. All biochemical results are presented as means and standard deviations ($N=9$).

CELL ISOLATION, MONOLAYER CULTURE, AND RNA EXTRACTION

To isolate chondrocytes, the cartilage pieces were incubated in Dulbeco's modified eagle's medium (DMEM, GIBCO, Grand Island, NY, USA) containing 0.2% collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA) and 5% fetal bovine serum (GIBCO) for 14–16 h at 37°C and 5% CO_2. The resulting cell suspensions were then filtered through 70 μm nylon filters (Cell Strainer; Falcon, Franklin Lakes, NJ, USA) and washed three times with phosphate buffered saline (PBS) containing 100 U/ml penicillin and 100 μg/ml streptomycin. The number and sizes of the isolated cells were then determined with a Z2 Coulter Counter and Size Analyzer (Beckman Coulter, Inc., Palo Alto, CA, USA). Total RNA for RT-PCR was isolated from two million cells from each of the three zones cells using the RNeasy Mini kit (Qiagen, Valencia, CA, USA).

After isolation, chondrocytes from the three zones were plated onto separate 10 cm tissue culture dishes at a density of 10 000 cells/cm^2. Cells were incubated at 37°C and 5% CO_2 in DMEM containing 10% fetal bovine serum, 0.4 mM proline, 50 μg/ml ascorbic acid, 10 mM HEPES, 0.1 mM non-essential amino acid, and 100 U/ml penicillin and 100 μg/ml streptomycin. Culture medium was changed twice weekly. When the cells reached 80–90% confluence, total RNA was extracted from cells in a single 10 cm culture dish.

GROWTH KINETIC STUDY

Chondrocytes from each layer were plated at a density of 2500 cells/cm^2 in 12-well culture plates. Cells were cultured for 12 days at 37°C and 5% CO_2, and medium was changed twice a week. At a specific time each day, cells from three wells were trypsinized and counted using a Z2 Coulter Particle Count and Size Analyzer. The number and size of cells were calculated as mean and standard deviation ($N=9$). Population doubling and population doubling time were determined using the following equation:$PD=3.32[\log(\text{cell\#}_{\text{harvested}})-\log(\text{cell\#}_{\text{plated}})]$.

RT-PCR

One microgram of total RNA per 20 μl reaction was reverse transcribed into cDNA using the SuperScript First-Strand Synthesis System (Invitrogen, Grand Island, NY, USA). One microliter of cDNA sample was subsequently amplified at an annealing temperature of 55°C for 35 cycles using the Takara Ex *Taq* DNA polymerase premix (Takara Bio Inc., Japan). Cartilage specific primers included type II collagen (F-gtggagcagcaagagcaagga, R-cttgccccacttacca gtgtg), aggrecan (F-gccttgagcagttcaccttc, R-ctcttctacgggg acagcag), COMP (F-caggacgactttgatgcaga, R-aagctggagc tgtcctggta), and type IX collagen (F-gtgttgctggtgaaaagggt, R-gggatcccactggtcctaattc). Two house-keeping genes, β-actin (F-tggcaccacaccttctacaatgagc, R-gcacagcttctcctta atgtcacgc) and GAPDH (F-gcctggtcaccagggctgc, R-tgct aagcagttggtggtgca) were used as an internal control. PCR products were separated by electrophoresis at 100 V on a 2% agarose gel in TAE buffer.

BIOCHEMICAL ASSAY OF SINGLE-LAYERED PHOTOPOLYMERIZING HYDROGELS

To compare the matrix synthesis in 3-D culture, chondrocytes from different zones were encapsulated in photopolymerizing gels. The hydrogel solution was prepared by mixing 10% weight/volume (w/v) of poly(ethylene glycol) diacrylate (PEGDA; Shearwater Corporation, Huntsville, AL, USA) in sterile PBS with 100 U/ml of penicillin and 100 μg/ml streptomycin (GIBCO, Invitrogen Corporation, Carlsbad, CA, USA). The photoinitiator, Igracure 2959 (Ciba Specialty Chemicals Corporation, Tarrytown, NY, USA), was added to the PEGDA solution and mixed thoroughly to make a final concentration of 0.05% w/v. Immediately prior to photoencapsulation, chondrocytes were resuspended in the solution to make a concentration of 20×10^6 cells/ml and were gently mixed to make a homogeneous suspension. One hundred microliters of cell/polymer/photoinitiator suspension was transferred into cylindrical molds with a 6 mm internal diameter and exposed for 5 min to long-wave, 365 nm UV light at 4 mW/cm^2 (Glowmark Systems, Upper Saddle River, NJ, USA). The hydrogels were then removed from their molds, and incubated in separate wells of 12-well plates. Culture medium was changed twice a week. After 3-week culture, ww and dw after 48 h of lyophilization were obtained from constructs from each zone ($N=9$). The dried constructs were crushed with a tissue grinder (Pellet Pestle Mixer; Kimble/Kontes) and digested in 1 ml of papain solution (Worthington Biochemical Corporation). DNA, GAG, and collagen assays were performed using the same methods described above. Results of GAG and collagen assays were normalized to DNA content.

CONSTRUCTION OF MULTI-LAYERED PHOTOPOLYMERIZING GELS

The steps to create multi-layered constructs are illustrated in Fig. 2. Briefly, 120 μl of the polymer solution containing lower chondrocytes (20×10^6 cells/ml) was placed in a 8 mm cylindrical mold and allowed to polymerize under the UVA lamp for 3 min (such that the solution only partially gelled), then 120 μl of polymer solution with middle chondrocytes (20×10^6 cells/ml) was added and exposed to UV light for 3 min. Finally, another 120 μl of polymer solution containing upper chondrocytes (20×10^6 cells/ml) was added and exposed to UV light for 4 min. The resulting multi-layered composite gels were removed from the mold and incubated in separate 12-well plates.

CELL TRACKING IN MULTI-LAYERED CONSTRUCTS

To confirm that the encapsulated cells stayed in the respective layer, cell tracking protocols (CellTracker™ Probes; Molecular Probes, Eugene, OR, USA) were performed 3 days after encapsulation according to the manufacturer's protocols. Briefly, the upper and lower chondrocytes were labeled by incubating for 30 min in 10 ml DMEM media with 5 μM CellTracker Green CMFDA. CellTracker Orange CMTMR was used for labeling the middle chondrocytes in the same way. Labeled cells were encapsulated to make multi-layered constructs in the same way as described above. Constructs were harvested for fluorescence microscopy immediately and 3 days after encapsulation. Fluorescence microscopy was performed using a fluorescein optical filter (485±10 nm) for

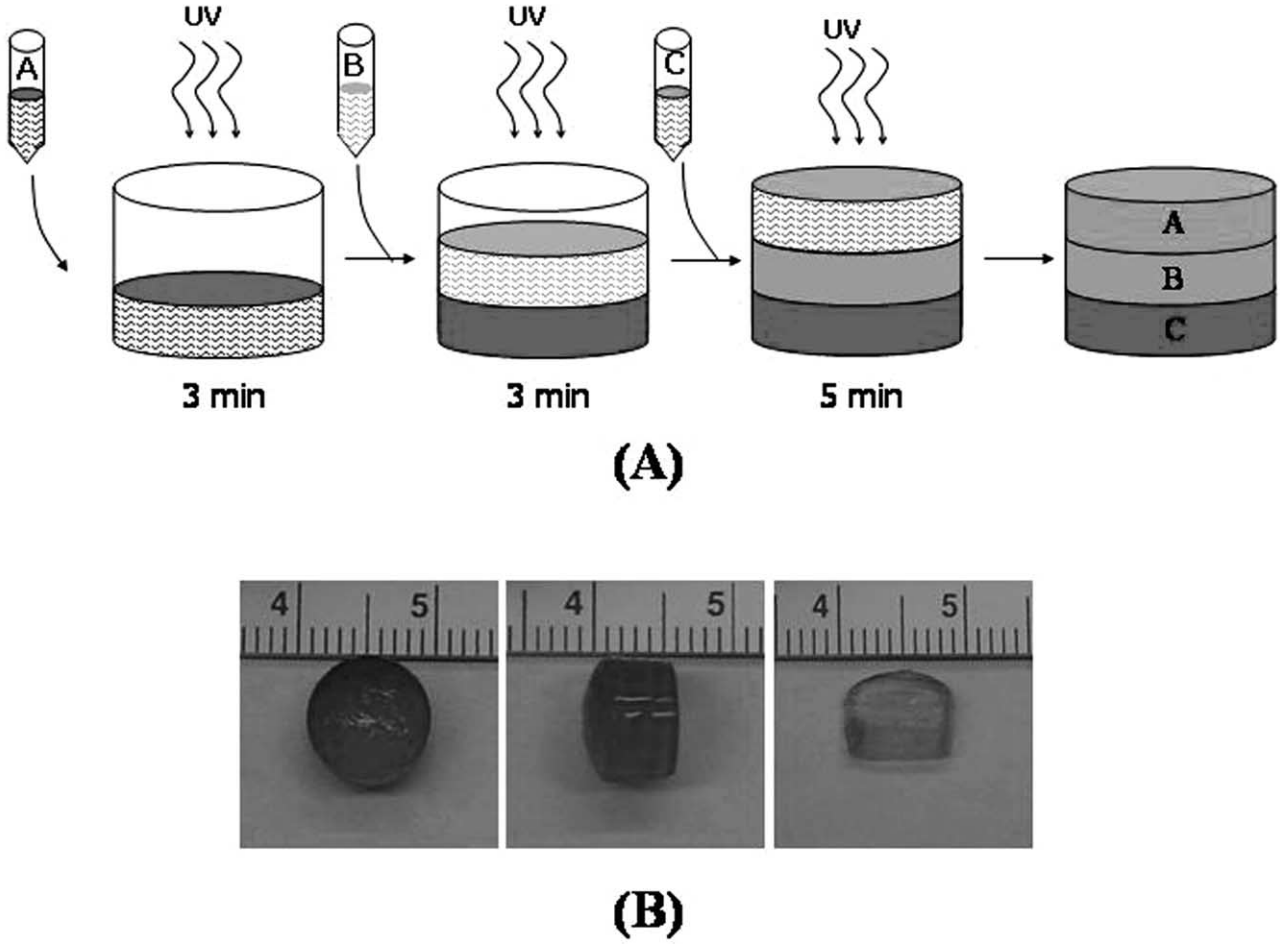

Fig. 2. Schematic drawings showing the technique to make multi-layered constructs: (A) encapsulating processes and (B) the produced constructs.

CMFDA and a rhodamine optical filter (530±12.5 nm) for CMTMR.

CELL VIABILITY ASSAY IN MULTI-LAYERED CONSTRUCTS

Cell viability of the encapsulated cells was evaluated with Live/Dead Viability/Cytotoxicity kit (Molecular Probes, Eugene, OR, USA). Briefly, thin slices (100–200 μm) of three layers were prepared with a surgical blade from the constructs after 3 and 21 days of culture. The slices were incubated for 30 min in Live/Dead assay reagents (2 μM calcein AM and 4 μM ethidium homodimer-1). Fluorescence microscopy was performed using a fluorescein optical filter (485±10 nm) for calcein AM and a rhodamine optical filter (530±12.5 nm) for ethidium homodimer-1.

HISTOLOGY AND IMMUNOHISTOCHEMISTRY

After 3-week culture, multi-layered constructs were harvested for histologic and immunohistochemical studies. The hydrogels were fixed overnight in 2% paraformaldehyde at 4°C and transferred to 70% ethanol until embedded in paraffin according to standard histological technique. Sections were stained with safranin-O/fast green. Immunohistochemistry was performed using the Histostain-SP kit (Zymed Laboratories Inc., San Francisco, CA, USA) following the manufacturer's protocol. Rabbit polyclonal antibody to type II collagen (Research Diagnostics Inc., Flanders, NJ, USA) was used as the primary antibody.

STATISTICAL ANALYSIS

Statistical analysis was performed with the SPSS (version 10.0; SPSS, Chicago, IL, USA) software package. Statistical significance of growth kinetic parameters and biochemical content was determined by ANOVA and post-hoc tests and set as $P<0.05$.

Results

HISTOLOGY OF CARTILAGE SLICES FROM DIFFERENT ZONES

Histologic evaluation confirmed that cartilage slices had been obtained from the upper, middle, and lower zones of the cartilage block (Fig. 3). The upper zone [Fig. 3(A and D)] had the highest cellularity, followed by the middle zone [Fig. 3(B and E)] and lower zone [Fig. 3(C and F)]. Cells of the upper zone were smaller than cells of the middle and lower zones. Cells along the articular surface of the upper zone showed flattened or ellipsoid-shaped morphology and

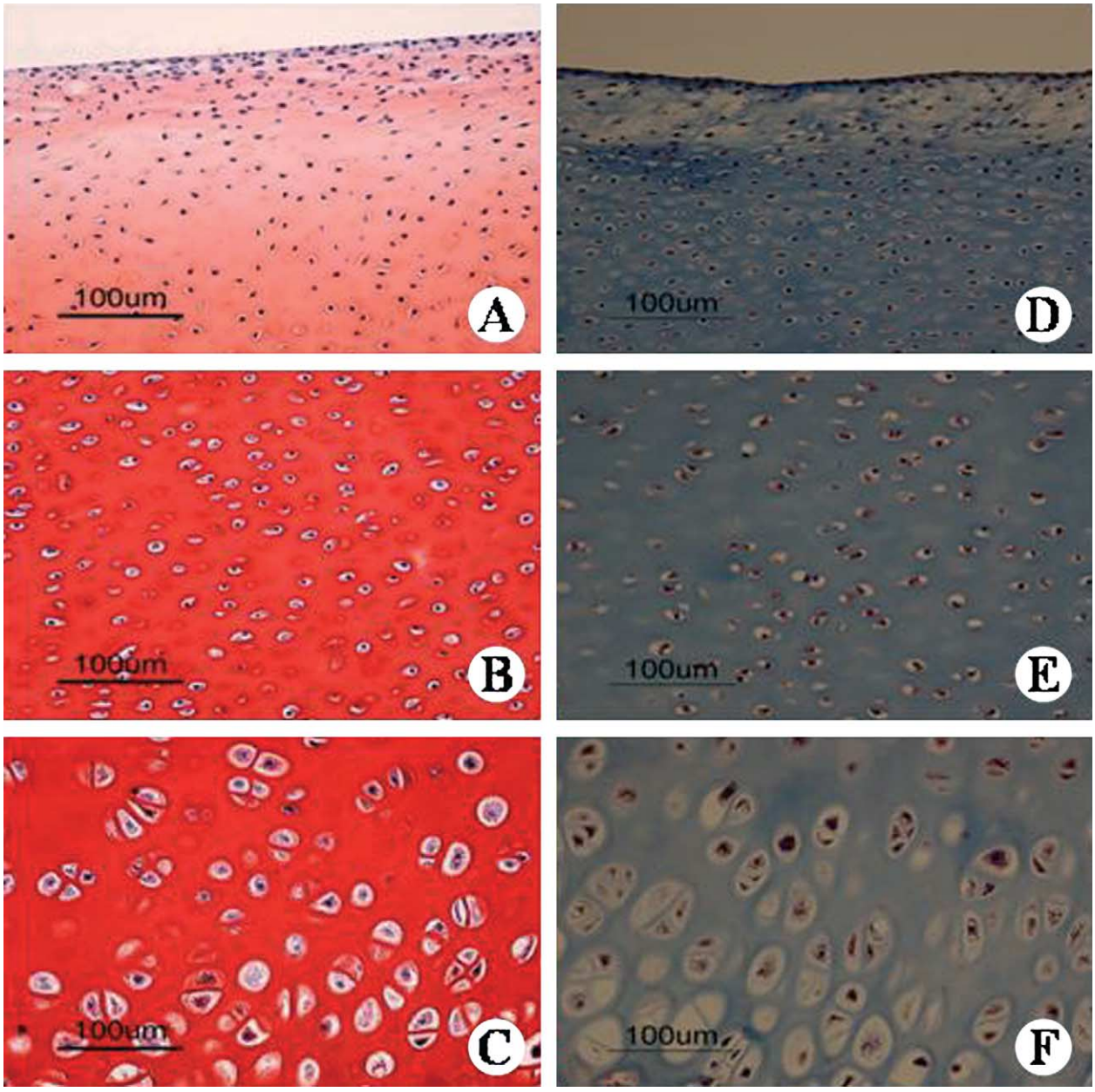

Fig. 3. Histology of cartilage slices confirmed that cartilage slices had been obtained from the three layers: upper (A, D), middle (B, E), and lower (C, F) zones (safranin-O staining (A–C), Masson's trichrome staining (D–F), 200×, meter bar=100 μm).

parallel arrangement with the articular surface. The intensity of safranin-O staining, indicating proteoglycan content, was the highest in the lower zone followed by the middle and upper zones [Fig. 3(A–C)]. The intensity of Masson's trichrome staining, directly related to collagen content, was the highest in the middle zone followed by the upper and lower zones [Fig. 3(D–F)].

BIOCHEMICAL ASSAY OF CARTILAGE SLICES FROM DIFFERENT ZONES

Results of the biochemical assays of cartilage slices from different layers were consistent with the histologic findings (Fig. 4). The water content was the highest in the upper zone [Fig. 4(A)]. The upper zone also had the highest DNA content, which was in line with the highest cellularity observed in the histologic examination [Fig. 4(B)]. GAG content of the lower zone was the greatest, followed by the middle and upper zones [Fig. 4(C)]. The middle zone had the highest collagen content followed by the upper and lower zones [Fig. 4(D)].

CELL NUMBER, SIZE, AND VIABILITY AFTER ISOLATION FROM DIFFERENT LAYERS

Assessment of cell number and size was performed in three experiments at different times ($N=3$ per each layer from three animals). Cell number and size were counted using a Z2 Coulter Counter and cell viability was determined by trypan blue dye exclusion method[36]. The greatest

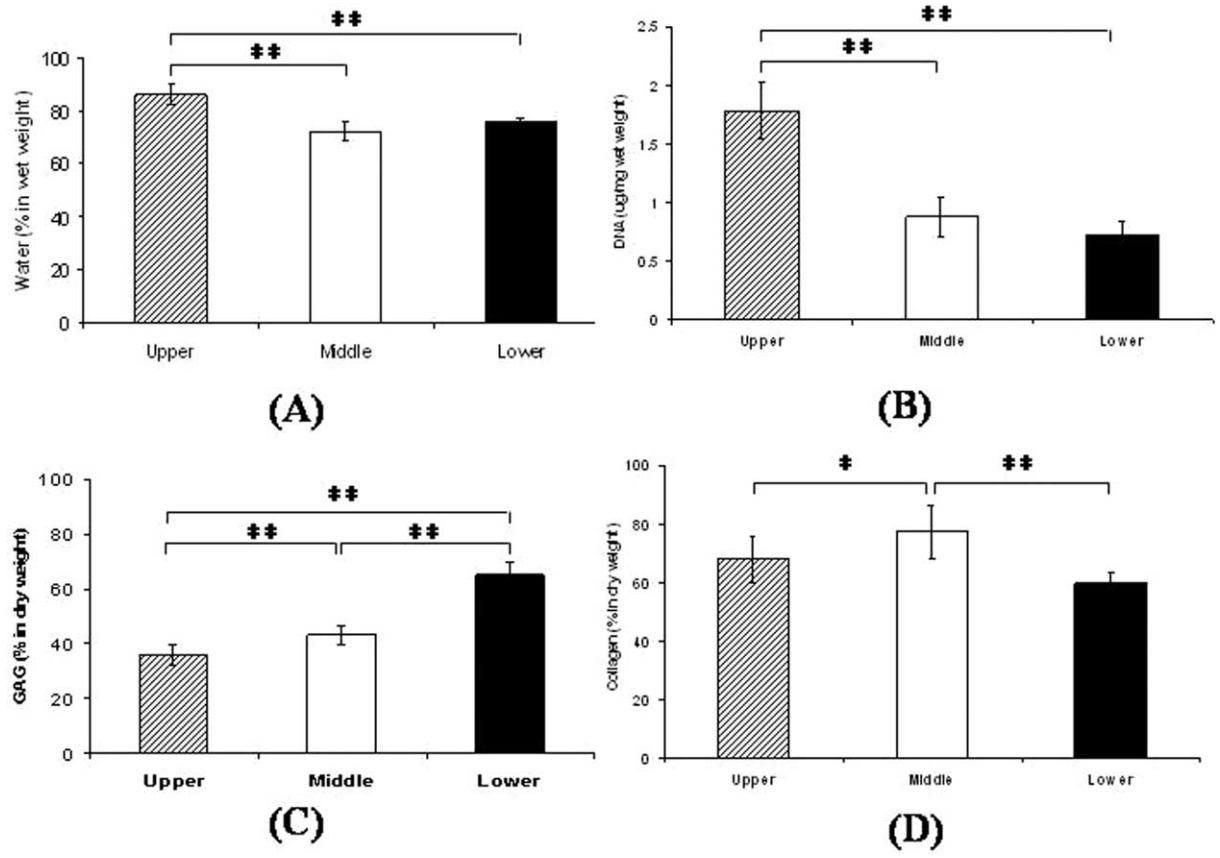

Fig. 4. Results of the biochemical assays of cartilage slices from different layers: water (A); DNA (B); GAG (C); and collagen (D) (*P<0.05 and **P<0.01).

number of cells per gram of tissue was obtained from the upper zone (42.7(±1.45)×10^6 cells per gram), followed by the middle zone (24.2(±2.57)×10^6 cells per gram) and the lower zone (13.2(±1.16)×10^6 cells per gram) (U vs M, P=0.000; U vs L, P=0.000; and M vs L, P=0.001). Cell sizes of the lower chondrocytes were the largest (diameter: 13.2±0.52 μm) followed by the middle chondrocytes (12.0±0.15 μm) and the upper chondrocytes (10.7±0.14 μm) (U vs M, P=0.005; U vs L, P=0.000; and M vs L, P=0.01). These quantitative measurements were consistent with histologic observations of chondrocytes in native articular cartilage (Fig. 2). The cell viabilities of chondrocytes from all three zones were greater than 97% and there was no difference among the three zones (P>0.05).

GROWTH KINETIC STUDY

When the primary isolated cells (P0) from each zone were cultured in monolayer, they demonstrated significant differences in growth kinetics (Fig. 5). The cells of the lower zone had the greatest proliferative capacity, as suggested by evaluation of the lag phase, population doubling time, and saturation density. The lower cells did not exhibit a lag phase of growth as the upper and middle cell populations [Fig. 5(A and C)]. The number of population doublings of the primary cells in the first 3 days of culture was the greatest in the lower cells (1.8), followed by the middle (0.8) and the upper (0.6) cells. There was no lag phase in the plated cells. During the exponential growth phase, the lower chondrocytes demonstrated a faster population doubling time (18.8±1.1 h) than the middle (22.4±0.9 h) and upper chondrocytes (26.1±1.1) (P=0.000 in all three comparisons: U vs M; U vs L; and M vs L) [Fig. 5(D)]. The differences in population doubling time among the three layers were maintained in the plated cells.

RT-PCR

The gene expression of the cartilage specific markers differed among the cells from different zones and the pattern of the changes with plating was also different (Fig. 6). Type II collagen expression of the upper chondrocytes was notably lower than the middle and lower chondrocytes. The aggrecan expression of primarily isolated cells had no remarkable differences among the zones and slight decreases were observed upon plating. In the primarily isolated cells, the expression level of type IX collagen of the lower cells was the strongest, followed by the middle and upper cells. This trend was maintained even upon plating. The gene expression of COMP was higher in the primarily isolated lower cells than in the upper and middle cells.

BIOCHEMICAL ASSAY OF SINGLE-LAYERED PEGDA HYDROGELS

Biochemical assays of single-layered PEGDA hydrogels revealed that the chondrocytes from each zone differed in

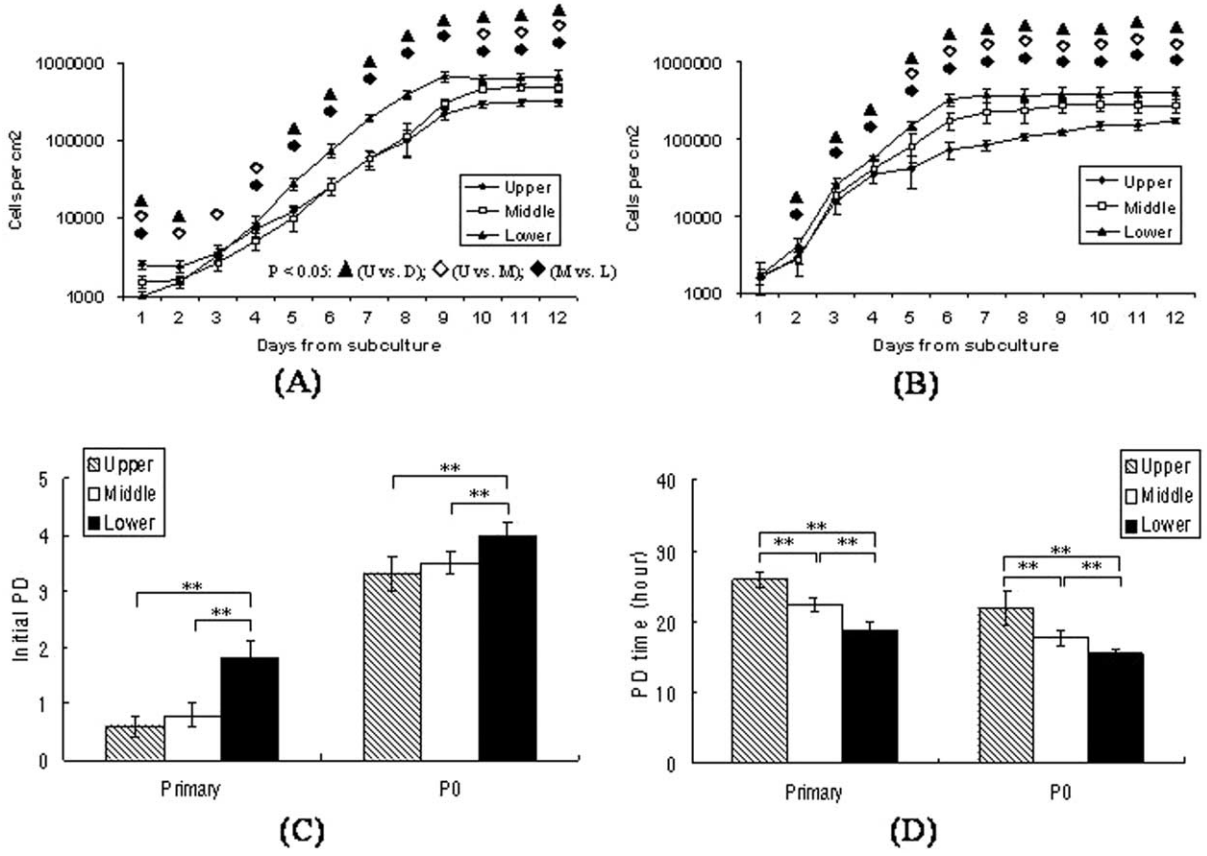

Fig. 5. Growth curves of the cells from different layers and the summary of the growth kinetic study. (A) Growth curves of primarily isolated chondrocytes. (B) Growth curves of passaged cells (passage, PO). (C) Initial population doublings defined as the number of population doubling for the first 3 days after plating. (D) Population doubling time (*P<0.05 and **P<0.01).

matrix synthesis even after 3-D culture (N=3). GAG synthesis by the middle and lower chondrocytes was significantly greater than that by the upper chondrocytes, by 26 and 46%, respectively. In addition, the lower chondrocytes synthesized 55 and 35% more collagen than the upper and middle chondrocytes, respectively.

CELL VIABILITY TEST IN MULTI-LAYERED CONSTRUCTS

Cell viability assay of multi-layered hydrogel constructs revealed that cells survived photoencapsulation and remained viable in tri-layered constructs that were approximately 8 mm thick (Fig. 7). No differences among the cells from different layers were found in cell viability after 3 [Fig. 7(A–C)] and 21 days of culture [Fig. 7(D–F)].

CELL TRACKING IN MULTI-LAYERED CONSTRUCTS

Cell tracking studies on the encapsulation day (data not shown) and 3 days after encapsulation confirmed that the encapsulated cells had stayed in the respective layer (Fig. 8). A small amount of cell settling was observed in the lower sections of the gels but there was no cell migration between the layers of the constructs from day 0 to 3.

HISTOLOGY AND IMMUNOHISTOCHEMISTRY OF MULTI-LAYERED CONSTRUCTS

Safranin-O staining revealed that each layer of multi-layered constructs showed similar histologic findings to that of the relevant zone of native cartilage [Fig. 9(A–C)]. The upper layer had small cells with a flattened or ellipsoidal cellular morphology whereas middle and lower layers had large cells with an oval or round cellular morphology. The diameter of pericellular matrix stained with safranin-O was greatest in the lower layer, followed by the middle and upper layers.

Immunohistochemisty for type II collagen showed that the location of collagen deposition was similar to that of proteoglycan synthesis shown in safranin-O staining. The diameter of positive staining pericellular areas was the greatest in the lower layer. Many cells in the upper layer had no positive staining in the pericellular regions [Fig. 9(D)].

Discussion

Zonal organization of articular cartilage is important for its normal function[14,15,17–20]. Chondrocytes play a crucial role in the generation and maintenance of the zonal architecture[14,15,17–20]. The experimental model presented

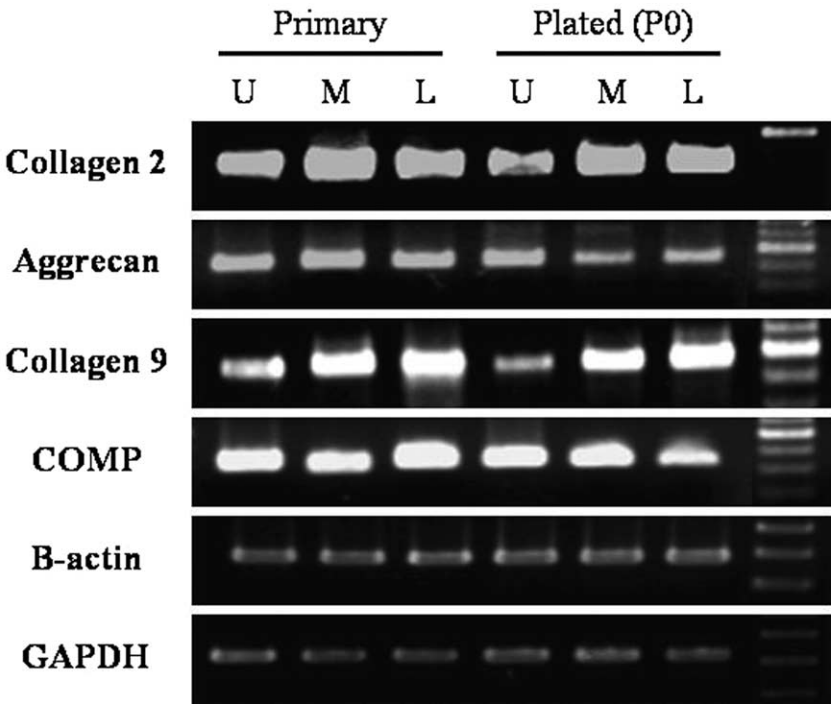

Fig. 6. RT-PCR of cartilage specific markers. β-Actin and GAPDH were displayed as the internal control. Abbreviations: U—upper; M—middle; and L—lower.

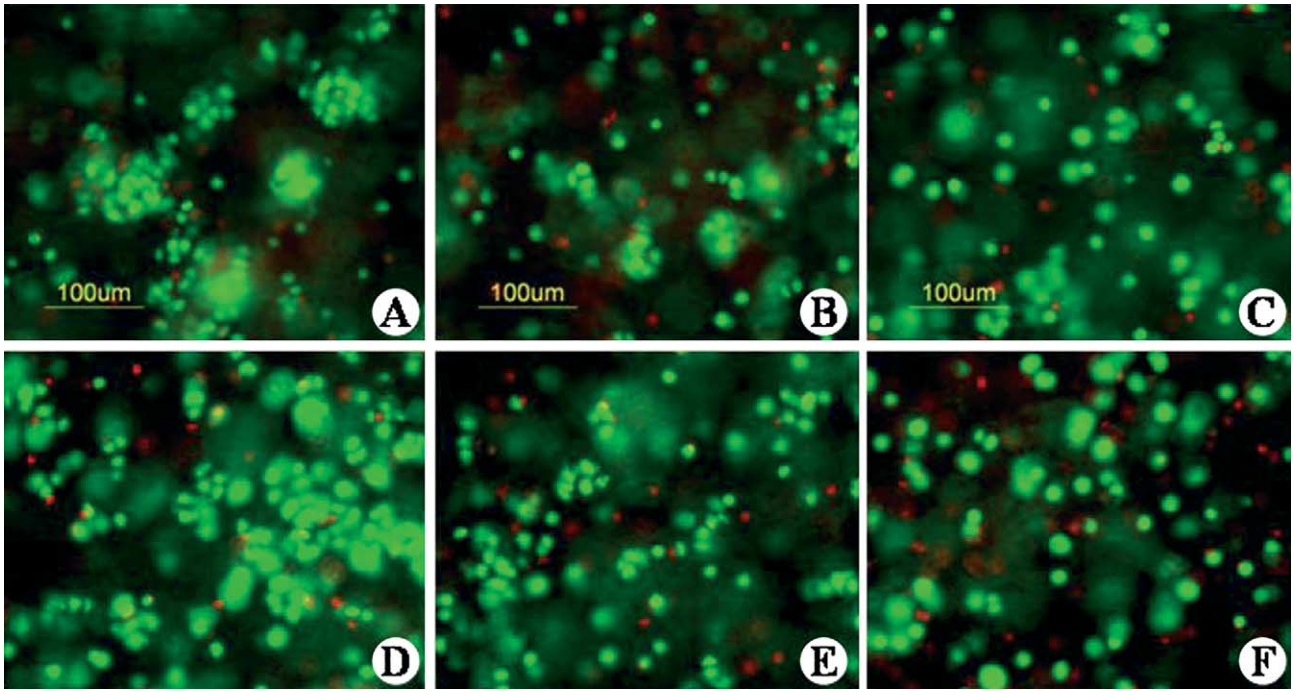

Fig. 7. Cell viability assay using the Live/Dead Viability/Cytotoxicity kit (Molecular Probes) performed at 3 (A–C) and 21 (D–F) days after plating: upper (A, D); middle (B, E); and lower (C, F) zones.

here can be used for studying the zonal organization of articular cartilage and for designing cell-based therapies to re-create zonal architecture in engineered cartilage tissue. The observations in histology and the findings relating to cell number, cell size, and matrix composition of the cartilage slices taken from each zone are consistent with previous studies[14–18], which validates our procedure to isolate tissue from the upper, middle, and lower zones. Study of the growth kinetics and gene expression of the different zonal chondrocytes in monolayer culture suggests

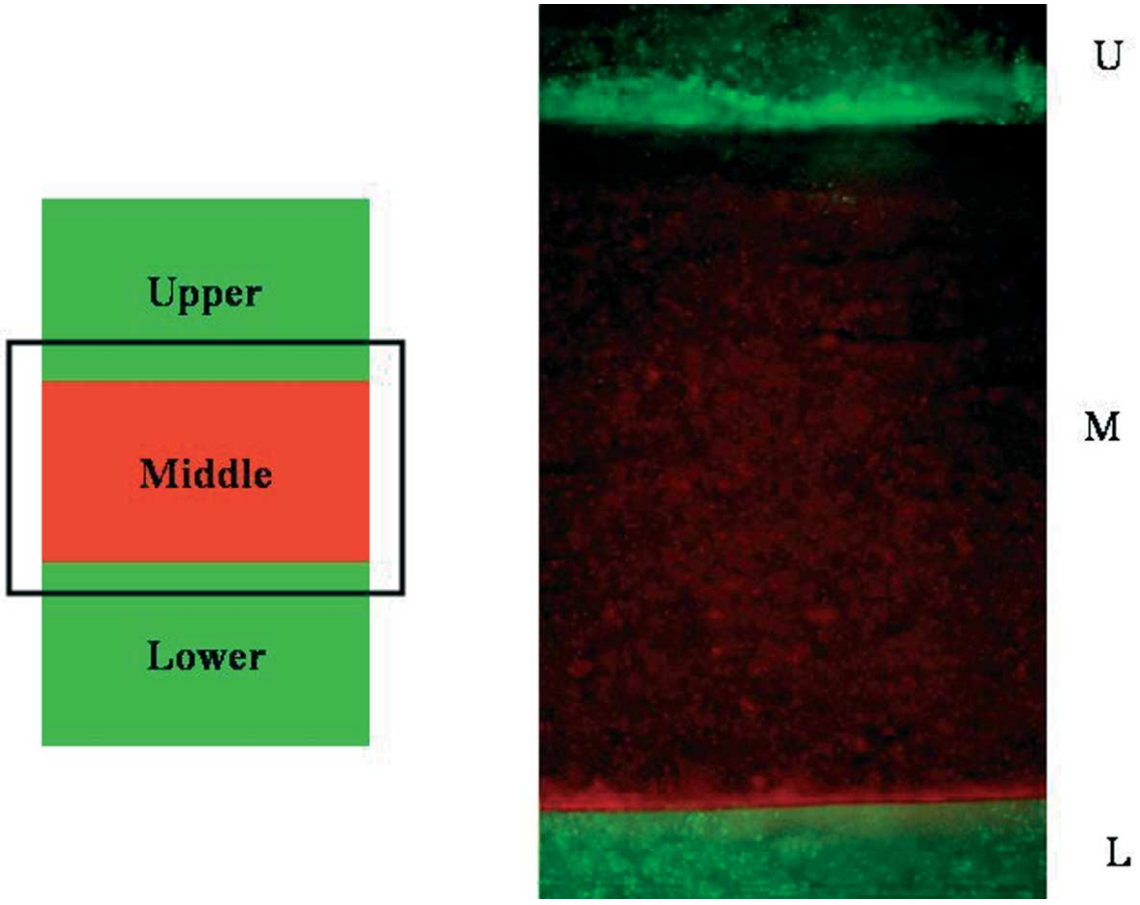

Fig. 8. Cell tracking demonstrating that the encapsulated cells stayed in the respective layers. Abbreviations: U—upper; M—middle; and L—lower.

that the chondrocytes continue to behave differently even when removed from their native environment. Indeed, analysis of GAG and collagen production in 3-D culture further demonstrates differences in matrix synthesis among the zones. This study demonstrates that the native zonal organization can be regenerated in engineered cartilage by utilizing the temporal and spatial control of the photopolymerization process and selective encapsulation of chondrocytes from different zones.

In this study, we used 5- to 8-week-old bovine legs and obtained cartilage slices from the distal femur. This bovine model has several advantages as an experimental model for studying the zonal organization. First, the knee joint is the structure to which cartilage tissue engineering is most commonly applied[3–5,7,8,11]. Since articular cartilage has its own specific anatomical and physical characteristics depending on the joint, studies with articular cartilage from the knee joint would be more relevant to cartilage tissue engineering applied to the same joint in humans. Second, thickness of a typical cartilage block from articular cartilage of distal femur ranged from 2 to 6 mm in 5- to 8-week-old calf, which offered considerable ease of dissection of the three zones. Histologic examination demonstrated that the articular cartilage used in this study had zonal architecture of articular cartilage (Fig. 3). Clear differences in the biochemical assays among the cartilage slices taken from different zones further support our hypothesis that cartilage slices taken by our method represent different zones of cartilage (Fig. 4). Taken together, the histology and biochemical assays indicate that our model satisfies the prerequisites for cell isolation and encapsulation in the multi-layered hydrogel system.

Despite these advantages, our model has limitations in applications to human articular cartilage. It is well known that articular cartilage from different species and subjects with different maturity vary in details of zonal organization and biochemical composition[4,14,16,37]. Our findings are influenced by the age and species choices. Despite the presence of zonal organization in the cartilage used in this study, the specimens had differences in details of histology from that of mature human articular cartilage. Mature human articular cartilage is typically thinner and has more complex organization than the cartilage used in this study[14,16]. It may be difficult to isolate homogenous chondrocytes from each zone of human articular cartilage. This difficulty is evidenced by the fact that all prior studies using human articular cartilage for this subject defined only two zones (superficial vs middle and deep zones)[17,38]. Given this difficulty with human articular cartilage, it is not surprising that most of previous studies used bovine or pig articular cartilage[17–19,23,39].

Although articular cartilage has a stratified structure, the difficulty of isolating chondrocytes from different zones has been noted by many authors[17,18,38]. This difficulty stems partly from the gradual nature of the zonal variation. Even though the top 20%, middle 60%, and bottom 20% of

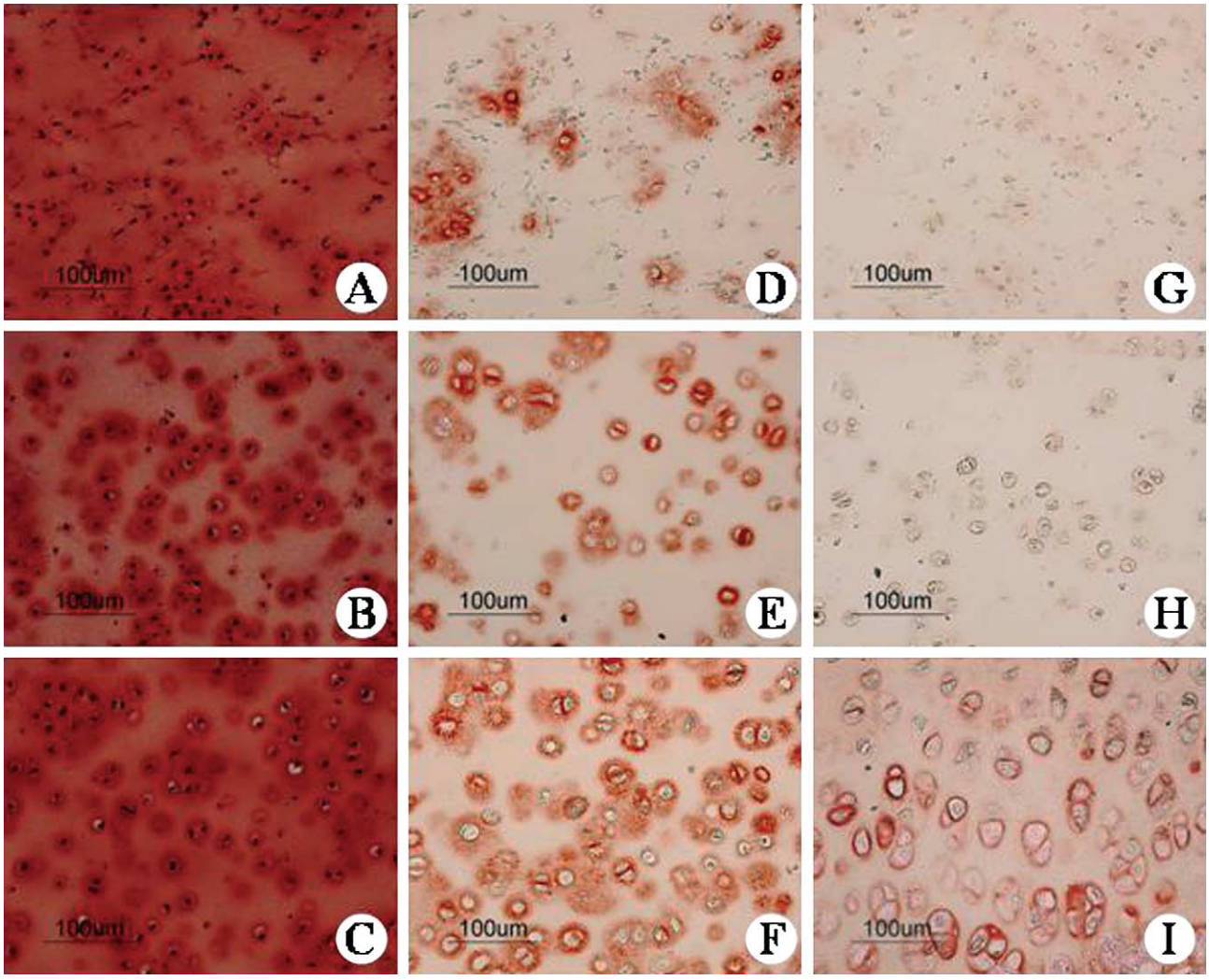

Fig. 9. Histology of a multi-layered PEGDA hydrogel encapsulated with different zone chondrocytes for different layers: safranin-O staining (A–C) and immunohistochemistry for type II collagen (D–I) (200×, meter bar=100 μm). Each layer of the constructs showed similar findings to that of native cartilage (A, D—upper; B, E—middle; and C, F—lower zones). Negative controls without primary antibody had no positive signals (G—upper zone, H—lower zone) and a positive control with a cartilage slice of lower zone (I).

articular cartilage are defined as the superficial, middle, and deep zones, respectively, the difference between adjacent zones may not be clearly distinguishable at a specific area[3,15,16,40]. In the model presented here, we tried to isolate three layers selectively by discarding intervening layers but we could not exclude the potential contamination by cells from the adjacent cells. To clarify that our definition of the three layers was different from the definition of the three (superficial, middle, and deep) layers of mature articular cartilage in previous studies, we have defined the three layers as 'upper, middle, and lower' zones.

Previous studies showed that articular chondrocytes retain the metabolic features characteristic of their zones of origin even after they are isolated from cartilage and cultured in suspension[18,19]. It was also found that chondrocytes from different zones have different growth kinetics when cultured in monolayer[17,21,39]. In line with these previous studies, the chondrocytes isolated by our method had different characteristics in cell proliferation and gene expression in 2-D culture. Lower chondrocytes had a greater proliferative capacity in monolayer, as suggested by their faster population doubling time compared with middle and upper cells in both P0 and P1 cultures (Figs. 4 and 5).

Our biochemical assays of the single-layered hydrogels indicate that cells from the different zones remained metabolically distinct when photoencapsulated and cultured in a hydrogel (Fig. 7). The environment of the hydrogel is similar to that of the native tissue in that the chondrocytes are isolated with little cell–cell contact, which encourages them to synthesize extracellular matrix[26–28,32]. The differences in biosynthetic activity, with the lower cells significantly exceeding the upper cells in terms of matrix synthesis, are in line with the study by Wong *et al.*[22]. They investigated the zone-specific biosynthetic activity in mature bovine articular cartilage and found significant differences between the biosynthetic activity of deep and superficial zones.

Photopolymerizable hydrogels are attractive scaffolds for tissue engineering applications since temporal and spatial control of the polymerization and gelation process is readily achieved. This control over gelation allows the creation of

more complex structures including multi-layered hydrogels. This study presents the technical feasibility for the photoencapsulation of chondrocytes from the three zones of articular cartilage such that the regional cellular variation found in the native tissue is re-created. The polymerization conditions and hydrogel properties support cell viability and matrix synthesis, as indicated by histological analysis. Tissue construct thickness resembling that of native cartilage was achieved without compromising cell viability or matrix production.

Our ultimate goal is to regenerate the structural, compositional, and mechanical properties of normal articular cartilage in engineered cartilage. Therefore, despite these encouraging results, the multi-layered constructs produced in this study need to be improved in several ways before they are considered engineered cartilage with clinical relevance. First, our multi-layered construct had a distinct interface separating the layers [Fig. 9(A)], which may act as a stress riser *in vivo*. Mechanical benefits of the multi-layered constructs were not evaluated in this study. Although articular cartilage is understood as a stratified structure, the distinction between the zones of the architecture is not discrete, but gradual. This gradual transition between the zones should be considered in improving the current method to generate multi-layered constructs. Second, mature human articular cartilage has more complex zonal organization. Regeneration of the calcified cartilage layer, which is typically found in normal mature articular cartilage, was not included in this model. Regeneration of the fine zonal organization needs to be considered for human application. Third, the particular photopolymerizing gel is not biodegradable. Ideally, scaffolds in engineered tissue should be able to degrade as new tissue is regenerated when transplanted. Further research is needed to investigate mechanical benefits of multi-layered constructs and to incorporate biodegradable properties into the photopolymerizing gel.

In summary, the general paradigm of tissue engineering strategies is to mimic as closely as possible the native environment and structure of a tissue in order to encourage the restoration of its structure and function. Restoration of the zonal organization in engineered cartilage tissue is an important consideration for these strategies. As an initial step towards this ultimate goal, we have presented an experimental model relevant to this subject. A method to isolate chondrocytes from different zones of young bovine articular cartilage is presented. We also show that cells from different layers have different behaviors in 2-D and 3-D cultures, which validates our surgical dissection method. Finally we have demonstrated the feasibility of re-creating the zonal characteristics using a multi-layered photopolymerizable hydrogel. We envision that this will greatly contribute to improvements in the field of cartilage tissue engineering.

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